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ORIGINAL ARTICLE ***Reproductive endocrinology***

Development and characterization of a novel long-acting recombinant follicle stimulating hormone agonist by fusing Fc to an FSH-β subunit

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STUDY QUESTION: Does a novel long-acting recombinant human FSH, KN015, a heterodimer composed of FSHα and FSHβ-Fc/Fc, offer a potential FSH alternative?

SUMMARY ANSWER: KN015 had *in vitro* activity and superior *in vivo* bioactivity than recombinant human FSH (rhFSH), suggesting KN015 could serve as a potential FSH agonist for clinical therapy.

WHAT IS KNOWN ALREADY: rhFSH has very short half-life so that repeat injections are needed, resulting in discomfort and inconvenience for patients. The longest-acting rhFSH available in clinics is corifollitropin alpha (FSH-CTP), but its half-life is not long enough to sustain the whole therapy period, and additional injections of rhFSH are needed.

STUDY DESIGN, SIZE, DURATION: Plasmids containing FSHα, FSHβ-Fc and Fc cDNA were transfected into Chinese hamster ovary (CHO) cells for KN015 production. The pharmacokinetics of KN015 was investigated in 6-week-old SD rats ($n = 6$/group) and healthy Cynomolgus monkeys in two different dose groups ($n = 2$/group). A series of experiments were designed for *in vitro* and *in vivo* characterization of the bioactivity of KN015 relative to rhFSH.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The purity and molecular weight of KN015 were determined by reducing and non-reducing SDS-PAGE. To measure KN015 half-life, sera were collected at increasing time points and the remaining FSH concentration was measured by enzyme-linked immunosorbent assay. To assess the bioactivity of KN015 versus rhFSH *in vitro*, firstly cAMP production was assessed in CHO cells expressing FSH receptor (FSHR) with the treatment of Fc/Fc, rhFSH or KN015 at eight different doses (0.03, 0.09, 0.28, 0.83, 2.5, 7.5, 22.5, 67.5 nM), and secondly cumulus oocyte complexes (COCs; $n = 20$/group) of ICR mice (primed-PMSG 44 h before sacrificed) were collected and cultured in medium containing 1.25 pM Fc/Fc, rhFSH or KN015 at 37°C and then germinal vesicle breakdown (GVBD) and COC expansion were observed at 4 and 16 h, respectively. The *in vivo* activity of KN015 was compared with rhFSH by ovary weight gain and ovulation assays. In the former, ovary weight gains in 21-day-old female SD rats, after a single subcutaneous injection of KN015, were compared with those after several injections of rhFSH over a range of doses ($n = 8$/group). Sera were harvested for estradiol (E2) analysis, and the ovaries were processed for hematoxylin and eosin (HE) staining, immunohistochemistry (IHC), TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labeling (TUNEL), RT–PCR and western blot. In the latter, 26-day-old female SD rats ($n = 8$/group) were injected with different doses of KN015 or rhFSH, and were sacrificed at 24 h after an injection of hCG (20 IU/rat). Moreover, the molecular responses stimulated by KN015 or rhFSH in the ovary were also analyzed through detecting expression of the FSH target genes (*Cyp19a1*, *Fshr* and *Lhcgr*) and phosphatidylinositide 3-kinase (PI3K) pathway activation.

MAIN RESULTS AND THE ROLE OF CHANCE: KN015 has a molecular weight of 82 kD and its half-life is 84 h in SD rats (10-fold longer than that of rhFSH) and 215 h in Cynomolgus monkeys. The EC50 value of the cAMP induction in CHO cells (KN015 versus rhFSH, 1.84 versus

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0.87 nM), COC expansion and oocyte maturation assays showed KN015 had approximately half of rhFSH's activity *in vitro*. A single dose of KN015 (1.5 pmol/rat, 166.1 ± 19.7 mg, $P < 0.01$) stimulated significantly larger ovary weight gain than several injections of rhFSH (1.5 pmol/rat, 59.3 ± 28.1 mg, $P < 0.01$). The serum E2 level in the KN015 group was significantly higher than that in rhFSH group. The number of oocytes obtained by ovulation induction was comparable with or higher in the KN015 group than in the rhFSH group. KN015 was more effective than rhFSH in inducing FSH target genes (*Cyp19a1*, *Fshr*, *Lhcgr*) or activating the PI3K pathway *in vivo*. Moreover, a single injection of KN015 promoted granulosa cell proliferation and prevented follicle atresia to the same extent as several injections of rhFSH.

LIMITATIONS, REASONS FOR CAUTION: All assays in this study were operated only in animals and clinical trials are needed to confirm they can be extrapolated to humans.

WIDER IMPLICATIONS OF THE FINDINGS: KN015 is a valuable alternative to FSH and may have great potential for therapeutic applications.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by National Basic Research Program of China (2011|CB944504, 2012CB944403) and National Natural Science Foundation of China (81172473, 31371449). The authors have no conflicts of interest to declare.

Key words: follicle growth / ovulation / follicle stimulating hormone / granulosa cell / oocyte

Introduction

FSH is a pituitary-secreted heterodimeric glycoprotein that is essential for follicle development in women and spermatogenesis in men (Pierce and Parsons, 1981; McGee *et al.*, 1997; Themmen and Huhtaniemi, 2000). Like other members of the glycoprotein hormone family (LH, thyroid-stimulating hormone and hCG), FSH consists of a common α subunit and a biologically specific β subunit. These two subunits bind to each other by a noncovalent bond. Proper subunit assembly is essential for FSH bioactivity and stability (Pierce and Parsons, 1981; Ryan *et al.*, 1988; Dias *et al.*, 2002).

In adult females, a cohort of immature follicles is cyclically recruited by FSH for their development. FSH promotes the development of immature pre-antral follicles to pre-ovulatory follicles, as characterized by the formation of a fluid-filled antrum within a follicle and the production of estrogen by granulosa cells (GCs) (McGee *et al.*, 1997). Estrogen is essential for the formation of secondary sex characteristics, dominant follicle selection, LH induction for ovulation and preparing the uterus for embryo implantation (Hunzicker-Dunn and Maizels, 2006).

By binding to its G-protein-coupled receptor (GPCR) that is exclusively expressed on granulosa cell (GC) surfaces, FSH induces adenylyl cyclase activity and, consequently, increases cAMP production (Sunahara *et al.*, 1996; Francis and Corbin, 1999). This elevated cAMP induces protein kinase A (PKA) activation, cAMP-responsive element binding protein (CREB) phosphorylation (Sassone-Corsi, 1998) and the transcription of various genes, including those for inhibin A (*Inha*) (Woodruff *et al.*, 1987), inhibin B (*Inhb*) (Turner *et al.*, 1989), FSH receptor (*Fshr*) (Tano *et al.*, 1997) and cytochrome P450 aromatase (*Cyp19a1*) (Orly *et al.*, 1980; Liu *et al.*, 2009). FSH also promotes the rapid activation of the phosphatidylinositide 3-kinase (PI3K) pathway in GCs, which results in the phosphorylation and activation of the downstream protein kinase B (AKT) (Gonzalez-Robayna *et al.*, 2000; Alam *et al.*, 2004; Fan *et al.*, 2008). The PI3K-AKT-Forkhead box protein O1 (FOXO1) pathway is vital not only for GC proliferation but also for inducing cyclin D_2 gene expression (Sicinski *et al.*, 1996; Fan *et al.*, 2008) and 'differentiation' target genes in GCs, such as *Cyp19a1* (Liu *et al.*, 2009), *Inhba*, epiregulin (*Ereg*) (Sekiguchi *et al.*, 2004; Wayne *et al.*, 2007) and LH receptor (*Lhcgr*) (Zeleznik *et al.*, 1974). It is also required for the inhibition of apoptosis by FSH in the GCs (Shen *et al.*, 2014).

Because FSH is the master controller of female reproduction, native or recombinant (modified) FSH has been used to treat female infertility for decades. The three currently commercially available recombinant hFSHs (rhFSH) are Gonal-F, Puregon and Elonva. Gonal-F and Puregon are produced using the same recombinant technology and have identical primary structures. They differ with regard to the glycosylation and purification procedures used, which results in a slightly more acidic Gonal-F when compared with Puregon, with an insignificant difference in their biological activities (Galway *et al.*, 1990; de Leeuw *et al.*, 1996). Because the *in vivo* half-life of an rhFSH is relatively short (8–10 h in rats and ~35 h in humans) (le Cotonnec *et al.*, 1994), a daily injection of rhFSH for 8–12 days is required to maintain follicle growth (Schaison *et al.*, 1993; Schoot *et al.*, 1994). Repeated injections are discomforting and inconvenient for patients. Thus, a longer-acting FSH would have advantages for clinical use.

FSH-CTP (INN Corifollitropin alpha), commercial name Elonva, has been approved as a sustained follicle stimulant (Beckers *et al.*, 2003). FSH-CTP is composed of a native FSHα subunit and a fusion of the C terminal fragment (CTP) of an hCG-β subunit, which is heavily O-glycosylated, to a human FSH β subunit (Fares *et al.*, 1992). FSH-CTP has a half-life of 17.3 h in rats and a 2- to 4-fold higher bioactivity when compared with rhFSH (Verbost *et al.*, 2011). In the clinic, a single subcutaneous injection of Elonva, albeit at a 10–15 fold higher dose than that used for wild-type (WT) FSH, may replace the first seven injections of a WT rhFSH preparation in a controlled ovarian stimulation (COS) treatment cycle (Duijkers *et al.*, 2002; Group CAD-FS, 2008). However, supplementation is still required with daily rhFSH for several days beginning from Day 8. Thus, various approaches are still being used to develop better, longer acting FSH by preparing structural modifications of WT FSH.

FSH-Fc is a long-acting FSH based on oral and pulmonary delivery (Low *et al.*, 2005). FSH-Fc molecules have been designed by linking an IgG1Fc domain to FSH, either as a single chain or as a heterodimeric form of FSH-Fc. The single chain FSH-Fc was a homodimer of FSHβ-α-Fc fusion whereas the heterodimer FSH-Fc was composed of α and β subunits separately fused with one Fc fragment. The half-lives of both FSH-Fc fusion proteins were between 55 and 210 h in Cynomolgus monkeys and 60 h in rats by oral delivery, which were longer than those of Elonva (Klein *et al.*, 2002). However, a single dose of FSH-Fc showed marginal *in vivo* bioactivity.

In this study, we generated a long-acting recombinant human FSH, KN015, with high bioactivity. KN015 was an FSH-Fc/Fc heterodimeric protein that was produced in CHO cells using recombinant DNA technology. KN015 consists of two noncovalently bond subunits: FSHα and FSHβ-Fc/Fc. The chain of single Fc domain of immunoglobulin G1 or the other chain of FSHβ-Fc folds independently to form FSHβ-Fc/Fc that is held together by a disulfide bond as indicated by the structure of IgG. The average molecular mass of KN015 was about 2.5 times greater than that of recombinant FSH (82 versus 32 kDa) and with much higher stability. Here, we investigated the metabolic clearance and bioactivity of heterodimeric FSH-Fc/Fc both *in vitro* and *in vivo*.

Materials and Methods

Ethical statement

All animal experiments were approved by Animal Care and Use Committee of the Zhejiang University School of Medicine (Hangzhou, China). All experiments protocols relative to Cynomolgus monkeys were conducted in accordance with the guidance of the Preclinical Safety Evaluation of Biotechnology-derived Pharmaceuticals S6 (R1) and the General Principles for the Technical Review of Non-clinical Safety of Biological Products for Treatment issued by China Food and Drug Administration (CFDA).

Animals

Immature 21- to 28-day-old female SD rats and ICR mice were obtained from the Zhejiang Academy of Medical Science, China. Healthy female Cynomolgus monkeys (*Macacafascicularis*) that were 3–4 years old were provided by Guangdong Landau Biotechnology Co., Ltd. (Guangzhou, China). The animals are housed with lights on at 06:00 and lights off at 18:00 and with temperature at 20–25°C. All animal experiments were performed in accordance with national guidelines for the care and use of laboratory animals.

The FSH-Fc/Fc heterodimer (KN015) expression and purification

A pcDNA4m expression vector came from a commercial pcDNA4/His-Myc vector (Invitrogen, USA) modification with site-directed substitution of a C for G at 3106, with a single *Pvu*II cloning site remaining. Then, *in vitro* synthetic sequences of FSH α-subunit and FSHβ-Fc cDNA, both flanked with *Hin*dIII (5′) and *Eco*RI (3′) sites, were cloned into a pcDNA4m vector, generating pcDNA4m-FSHα and pcDNA4m-FSHβ-Fc, respectively. A sequence encoding a 15-amino acid peptide (GGGGSGGGGSGGGGS) was added between the FSHβ and Fc sequence. A 1.6-kb FSHα fragment digested by *Bgl*II-*Pvu*II from pcDNA4m-FSHα was inserted into pcDNA4m-FSHβ-Fc, which was digested by *Bgl*II and *Nru*I prior to ligation, generating pcDNA4m-FSH-Fc. The Fc gene was amplified by PCR with an IgG1 signal peptide sequence and a 5-amino acid (GSGGG) linker sequence at the 5′ terminal end, and was sub-cloned into a pcDNA4m vector (designated pCDNA4m-Fc). A 1.8-kb Fc fragment by *Nru*I-*Pvu*II digestion from pcDNA4m-Fc was ligated with an *Nru*I digested pcDNA4m-FSH-Fc plasmid. The resulting plasmid (pcDNA4m-FSH-mono) contained three cytomegalovirus promoters and genes encoding for FSHα, FSHβ-Fc and Fc. A schematic for plasmid construction is shown in Fig. 1A.

Chinese hamster ovary (CHO) cells were transfected with pcDNA4m-FSH-mono using Lipofectamine 2000 (Invitrogen, USA). Twenty-four hours after transfection, cells were split into 100 mm dishes and fed with selection medium (Dulbecco's modified Eagle's medium [DMEM], 10% (v/v) fetal bovine serum and 200 μg/ml zeocin). Fresh selection medium was replaced every 3 days and 6 weeks later and mono-clones were picked and seeded in a 24-well plate for amplification. Fc in the supernatant was detected with an Fc enzyme-linked immunosorbent assay (ELISA) kit and FSH levels were determined with a Human FSH ELISA kit (DRG, Germany). The percentage of FSH-Fc/Fc could be calculated directly from non-reducing SDS-PAGE since the molecular weight of FSH-Fc/FSH-Fc, Fc/Fc homodimers and FSH-Fc/Fc is different. Cells that expressed high FSH-Fc/Fc heterodimer levels, but low levels of FSH-Fc/FSH-Fc and Fc/Fc homodimers were selected for FSH-Fc/Fc heterodimer production on a large scale.

The culture medium that contained FSH-Fc/Fc heterodimers was purified using immunoaffinity chromatography with FcRn, and further purified with Source Q (GE Healthcare, USA) to remove FSH-Fc/FSH-Fc and Fc/Fc homodimers. Briefly, the FcRn eluent (adjusted to pH 8.0) was loaded into a Source Q chromatography column and gradient eluted with buffer B (10 mM Tris–HCl, pH 8.0, 200 mM NaCl) to collect FSH-Fc/Fc heterodimers. Ion exchange chromatography eluent fractions (1 ml) were collected in tubes that contained 4 ml of 50 mM sodium phosphate buffer (pH 7.5) and further diluted with water for concentration and analysis. FSH-Fc/Fc heterodimer purity was >98% by size exclusion high performance liquid chromatography (SE-HPLC) and Coomassie blue staining after reducing and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). FSH-Fc/Fc heterodimer was designated KN015.

cAMP induction assay *in vitro*

A CHO cell line that had been stably transfected with the human FSH receptor gene was used to detect cAMP production in response to KN015 stimulation. cAMP levels were determined using a cAMP-Screen cAMP Immunoassay System (Life Technology, USA) according to the manufacturer's protocol in a Hewlett-Packard Topcount. Cells were cultured in 96-well plates (2×10^4 cells/well) with 100 μl of DMEM/F-12 (Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma, USA) for 24 h. Fc/Fc, recombinant hFSH (Gonal-F, Merck Serono, Germany) and KN015 were serially diluted (starting at 67.5 nM with 1:3 dilutions) in DMEM/F12. Cells were stimulated for 1 h (37°C), and were then lysed to determine cAMP levels. In each experiment, every dilution point was tested in three wells of cells and the experiment was repeated three times.

KN015 pharmacokinetics

KN015 pharmacokinetic parameters with Gonal-F used as a control were determined after administering single subcutaneous doses of 3.8 or 38 μg/kg body weight in 6-week-old female SD rats ($n = 6$/group). Blood samples were obtained from all rats at intervals 0–192 h after injection. Sera were prepared by allowing the blood to clot for 2 h at room temperature, and then centrifuging for 20 min at 845 *g*. Rat serum FSH concentrations were determined using a Human FSH ELISA kit (DRG, Germany). KN015 levels in rat serum were determined by sandwich ELISA using an anti-FSH antibody (Fitzgerald Industries, USA) and a horse radish peroxidase-anti-Fc antibody (LakePharma, USA).

Healthy female Cynomolgus monkeys that were 3–4 years old and with body weights of 3.03–3.27 kg were selected for KN015 pharmacokinetic assays using different administration routes: subcutaneous (sc.) and intravenous (iv.) injections. Prior to administration, monkeys were anesthetized with a combination of ketamine and valium. Administered doses were 38 μg/kg body weight of KN015 (in 0.9% (m/v) NaCl, pH 7.4) for intravenous injections and 3.8 and 38 μg/kg for subcutaneous injections. Blood samples were obtained at different time points (0–672 h) after injections, and KN015 levels remaining in sera were measured as described earlier.

Ovarian weight gain assay and serum estradiol (E2) levels

Female SD rats (21 days old) with body weights of approximately 50 g were subcutaneously injected on Day 1 with increasing doses of FSH (either

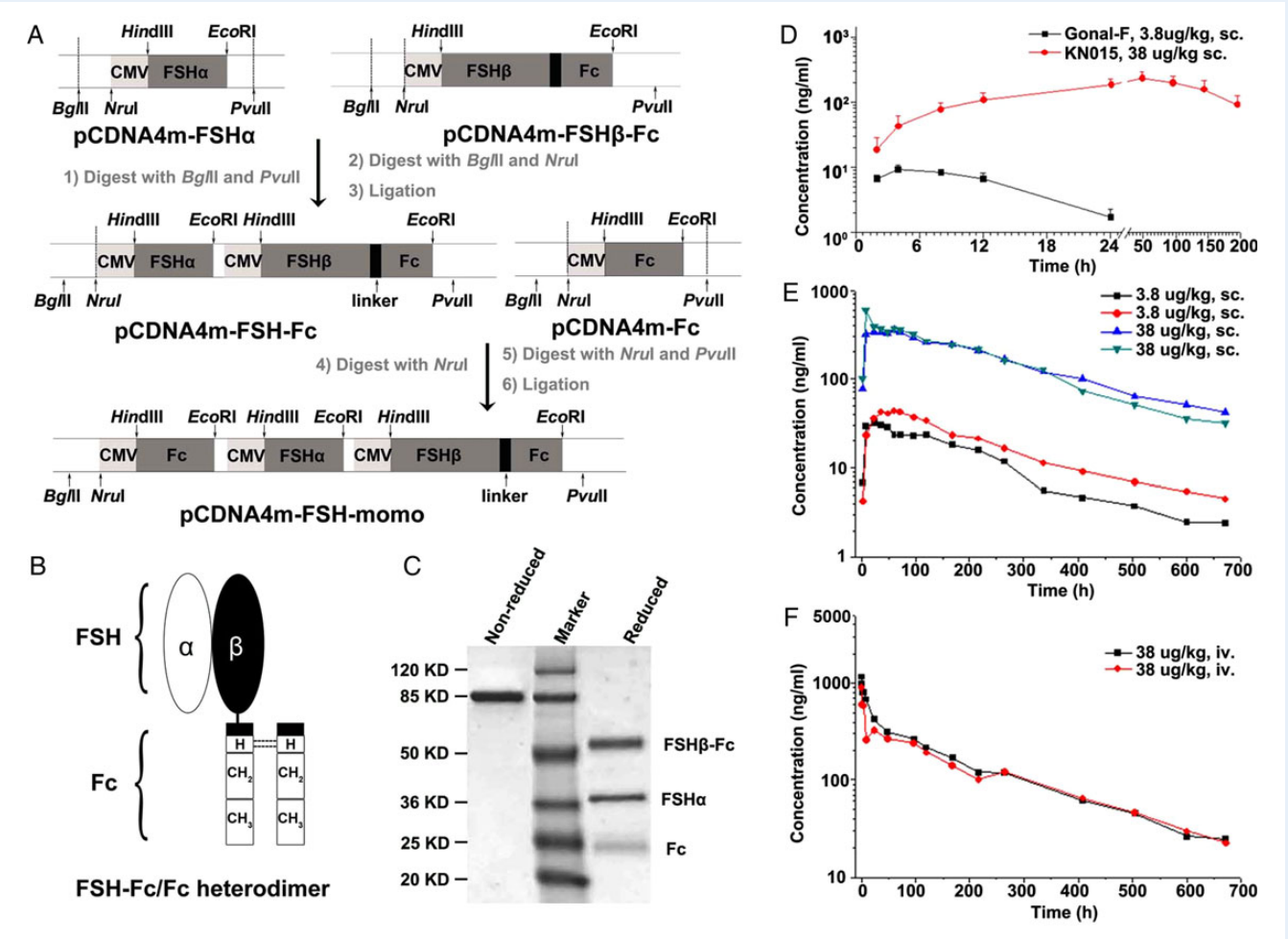


Figure 1 KN015 generation and *in vivo* half-life determinations with rats and Cynomolgus monkeys. (**A**) Heterodimer FSH-Fc/Fc (KN015) construction. A *Bgl*II-*Pvu*II fragment of FSHα was ligated with a *Bgl*II-*Nru*I fragment that included FSHβ-Fc. The *Nru*I-*Pvu*II fragment of Fc was inserted into *Nru*I-enzyme prepared fragment of FSH-Fc, which generated the heterodimer FSH-Fc/Fc, designated KN015. (**B**) Schematic of the KN015 protein. (**C**) SDS-PAGE analysis of KN015 under non-reducing and reducing conditions. (**D**) Pharmacokinetics using immature rats after a single subcutaneous (sc.) injection of 38 μg/kg of KN015 or 3.8 μg/kg of Gonal-F (means ± SEM's; $n = 4$/time point). (**E**) Pharmacokinetics using Cynomolgus monkeys after a single subcutaneous (sc.) injection of 3.8 or 38 μg/kg of KN015. (**F**) Pharmacokinetics using Cynomolgus monkeys after a single intravenous (iv.) injection of 38 μg/kg of KN015. Serum KN015 concentrations were determined using an FSH/Fc sandwich ELISA. In (E) and (F), each curve indicates results for one monkey.

heterodimer FSH-Fc or KN015 given once, but Gonal-F given seven times) plus hCG (r-hCG, Merck Serono, Germany) in a modified Steelman–Pohley test (Steelman and Pohley, 1953). The heterodimer FSH-Fc (0, 0.375, 0.75 or 1.5 pmol/injection; $n = 8$ per group) or KN015 (0, 0.19, 0.375, 0.75, 1.5, 3 or 6 pmol/injection; $n = 8$ per group) plus hCG (3.75 IU/rat) was subcutaneously injected only on Day 1, and on the subsequent 3 days. Animals received once injection of hCG (3.75 IU/rat) every day only (at 24, 48 and 72 h). The Gonal-F was administered (0, 1.5, 3 or 6 pmol/injection; $n = 8$ per group) every 12 h for seven times over 4 days, and on every day only one dose of hCG (3.75 IU/rat) was injected (at 0, 24, 48 and 72 h). Because the half-life of KN015 was much longer than that of Gonal-F, KN015 was used once, whereas Gonal-F was given every 12 h until rats ($n = 8$/group) were sacrificed and ovarian weights were determined at 84 h after the first injection. Sera were prepared as described earlier and stored at −20°C for E2 measurements.

Serum E2 levels were determined with a solid-phase, ligand-labeled, electrochemiluminescent enzyme-linked immunoassay using a Cobas®4000 automated random access immunoluminescence analyzer (Roche, Switzerland).

Rat ovulation induction

Immature (26–28 days old) female SD rats were treated with Gonal-F or KN015 to stimulate folliculogenesis. Animals received a single injection of KN015 (1, 2, 3, 6 and 12 pmol/injection; $n = 8$/group) or four injections of Gonal-F (1, 2, 3, 6 and 12 pmol/injection; $n = 8$/group) at 24 h apart. At 84 h after the first injection, a single injection of hCG (20 IU) was administered to induce ovulation. As a negative control, some rats ($n = 8$) were treated with hCG alone. Rats were sacrificed at 24 h after the hCG injection. Ovulated oocytes were harvested from oviducts and their numbers were recorded.

In vitro germinal vesicle breakdown and cumulus oocyte complex expansion assay

In vitro germinal vesicle breakdown (GVBD) and cumulus oocyte complex (COC) isolation and culture were previously described (Fan *et al.*, 2010). Briefly, fully grown, non-expanded COCs were harvested from ovaries of

5 IU pregnant mare serum gonadotrophin (Ningbo Sansheng, China) primed immature ICR mice. COCs and GCs were released from antral follicles by puncturing the ovaries. COCs were collected by pipetting, pooled and treated as previously described. COCs (≈30) were plated in 100 μl of defined COC medium (minimum essential medium [MEM], 25 mM HEPES, pH 7.4, 0.25 mM sodium pyruvate, 3 mM L-glutamine, 1 mg/ml of BSA, 100 IU penicillin/ml, 100 μg streptomycin/ml) with 1% (v/v) FBS containing vehicle, or 1.25 pM Fc/Fc, Gonal-F or KN015 and cultured overnight with 20 ng/ml EGF as a positive control. Sixteen hours after culture, COC expansion was examined and photographed by a stereomicroscope (SMZ1500, Nikon, Japan). For oocyte GVBD assay, COCs ($n = 20$, 3 repeats) were cultured in the COC medium containing different doses of Fc/Fc (0.3, 0.6, 3, 6, 30, 60, 300 or 600 nM), Gonal-F (0, 0.3, 3, 30 or 300 nM) or KN015 (0, 0.6, 6, 60 or 600 nM) and GVBD rates were assessed at 4 h after culture. For RT–PCR analysis, 50 COCs were harvested after culture for 4 h with vehicle, Gonal-F or KN015.

Granulosa cell culture

GCs were harvested from estradiol-primed (48 h) 21-day-old SD rats. Briefly, undifferentiated GCs were released from pre-antral follicles by puncturing with a 26.5 gauge needle. Cells were cultured (1×10^6 cells/ml) in DMEM/F12 medium supplemented with 5% (v/v) FBS (Gibco, USA), 100 IU penicillin/ml and 100 μg streptomycin/ml (Gibco, USA) in 12-well or 96-well plates. After overnight culture, cells were washed and cultured in serum-free medium for 12 h before adding FSH. GCs in 12-well plates had increasing concentrations of KN015 or Gonal-F added (0, 12, 120, 300, 600, 1200, 2400 or 4800 pM), and were harvested for RNA extraction and western blotting at the indicated time points after FSH treatment. GCs in 96-well plates had 1200 pM KN015 or Gonal-F added for 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays at 24, 48 and 72 h after treatment.

RNA isolation and real-time RT–PCR

Total RNA was extracted from rat ovaries or GCs using Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions. First-strand cDNAs were created from 2 μg of total RNA by Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, USA). Real-time PCR analyses were conducted using Q Tag SYBR Green Master Mix (ABI, USA) and an Applied Biosystems 7500 real-time PCR system. The expressions of *Fshr*, *Cyp19a1* and *Lhcgr* were determined by normalizing to endogenous β-actin mRNA levels; relative expression was calculated using the comparative Ct. For each indicated gene, the relative transcript level of the control sample (left-hand bar in each graph) was set to 1. Relative transcript levels of the other genes were compared with the control, and fold-changes are shown on the *Y*-axes. For each experiment, quantitative PCR was done in triplicate. Primer sequences used are presented in Table I.

TUNEL assay

Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assays were performed for 10% (v/v) formalin-fixed paraffin-embedded sections using an ApopTag Plus peroxidase *in situ* apoptosis detection kit (s7101, Millipore, USA), according to the manufacturer's instructions.

Histology

Paraffin-embedded sections of rat ovaries from the ovary weight gain assay were stained with HE or immunohistochemistry (IHC) as previously described (Fan *et al.*, 2010), using the anti-3β-hydroxysteroid dehydrogenase (HSD) (1:200, sc-30820, Santa Cruz, USA) and anti-phospho-histone H3 (1:200, ab-1173-1, Abcam, UK) followed by reacting with biotin-labeled secondary antibodies (VECTASTAIN ABC kit, Vector Laboratories, USA) for 30 min and 3,3′-Diaminobenzidine (DAB) peroxidase substrate kit (SK-4100, Vector Laboratories, USA) for development. Sections were visualized and photographed by a Nikon microscope (Eclipse 80i, Japan).

Table I Sequences of RT–PCR primers.

Gene name	Species	Primer sequences (5′-3′)	PCR product size (base pairs)
Areg	Mouse	F: 5′-GAGGCTTCGACAAGAAAACG-3′ R: 5′-TTTATCTTCACACATCTCTTTATGTACAG-3′	210
Ereg	Mouse	F: 5′-ACACTGGTCTGCGATGTGAG-3′ R: 5′-GTCCCCTGAGGTCACTCTCTC-3′	197
Btc	Mouse	F: 5′-CTCCCTCCTGCATCTGTGAG-3′ R: 5′-CTCTTGAATATCTTCACTTATGGGAG-3′	251
Has2	Mouse	F: 5′-GTTGGAGGTGTTGGAGGAGA-3′ R: 5′-ATTCCCAGAGGACCGCTTAT-3′	155
Ptgs2	Mouse	F: 5′-TGTACAAGCAGTGGCAAAGG-3′ R: 5′-CCCCAAAGATAGCATCTGGA-3′	230
Tnfaip6	Mouse	F: 5′-TTCCATGTCTGTGCTGCTGGATGG-3′ R: 5′-AGCCTGGATCATGTTCAAGGTCAAA-3′	328
Ptx3	Mouse	F: 5′-GTGGGTGGAAAGGAGAACAA-3′ R: 5′-GGCCAATCTGTAGGAGTCCA-3′	190
Fshr	Rat	F: 5′-ACCATGGCTTAGAAAATCTGAAG-3′ F: 5′-TGATCCCCAATTTGAGTCATATC-3′	226
Cyp19a1	Rat	F: 5′-TCATCTTCCATACCAGGTCCTG-3′ R: 5′-TCCACTGATCCAGACTCTCATG-3′	144
Lhcgr	Rat	F: 5′-GCAGATAACGAGACGCTTTATTC-3′ R: 5′-ACGACTGGTCAGGAGAACAAAG-3′	234
Actin	Mouse and rat	F: 5′-GCTCTTTTCCAGCCTTCCTT-3′ R: 5′-GTACTTGCGCTCAGGAGGAG-3′	234

Western blot analysis

Rat ovaries were homogenized in cold cell lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% (v/v) TritonX-100, 10 mM NaF, 1 mM EDTA) and centrifuged (12000 *g*, 15 min, 4°C) for whole cell extract preparation. After the protein concentration was measured, the whole cell extracts were dissolved in sodium dodecyl sulfate (SDS) sample buffer (63 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (m/v) SDS, 0.0025% (m/v) bromophenol blue and 5% (v/v) β-mercaptoethanol). Protein lysates (30 μg of total protein per lane) were boiled, separated by SDS-PAGE and then electrophoretically semi-dry transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Membranes were blocked with 5% (m/v) skim milk and incubated with anti-pERK1/2 (#9101, cell signal technology, USA), anti-pFOXO1 (#9464, cell signal technology, USA), anti-pCREB (#9191, cell signal technology, USA), anti-pAKT (#4060, cell signal technology, USA) and anti-ERK1/2 (SC-93, Santa Cruz, USA) antibodies. All antibodies were used at 1:1000 dilutions in blocking buffer. After probing with primary antibodies, membranes were washed with Tris–HCl buffered saline that contained 0.05% (v/v) Tween-20 (TBST), and then incubated with a goat anti-rabbit (1:5000, #111035003, Jackson Immunoresearch Laboratories) HRP-conjugated secondary antibody. Finally, target protein bands were detected using a Super Signal West Pico Chemiluminescent Substrate (ECL, Thermo Scientific, USA).

Statistical analysis

The effect of KN015 on induction of cAMP in CHO cells was analyzed to fit to a four-parameter logistic function by nonlinear least squares analysis using MicroCal Origin (Northampton, USA) to determine EC50 values. The pharmacokinetic parameters collected from SD-rats or Cynomolgus monkeys were estimated by a non-compartmental model (NCA) method using WinNonlin version 6.2 (Pharsight). Area under the serum concentration curve ($AUC_{0\text{-}inf}$) was calculated for the subcutaneous (sc.) and intravenous (iv.) phases. The absolute bioavailability of KN015 was determined as ($AUC_{0\text{-}inf}$ sc.)/($AUC_{0\text{-}inf}$ iv.). The two-way ANOVA add-in in Excel was applied to the ovary weight gain assay and E2 serum levels. The dose–response curves and EC50 were calculated by Graphpad Prism using a four-parameter logistic equation: $Y = \text{MIN} + ((\text{MAX} - \text{MIN})/(1 + 10^{(\log\ (\text{EC50}) - X)})\text{Hillcoefficient})$. A one-way analysis of variance (ANOVA) was used for analyzing the data obtained from RT–PCR, ovulation induction assay, MTT assay and TUNEL assay. Following a one-way ANOVA, *t*-test ($P < 0.05$ as significant) was used to determine whether there was significant difference in the ovulation induction number or atresia follicle number between KN015 and Gonal-F in every dose-matched group.

Results

KN015 (FSH-Fc/Fc heterodimer) expression and characterization

KN015 is designed as a heterodimer FSH-Fc/Fc that consists of two parts: FSHα and FSHβ-Fc/Fc (Fig. 1B), and was constructed by FSHα, an Fc fragment of an IgG1 molecule (containing the hinge region and CH2 and CH3 domains) and FSHβ-Fc fusion (Fig. 1A). The chain of single Fc domain of immunoglobulin G1 or the other chain of FSHβ-Fc folds independently to form FSHβ-Fc/Fc that is held together by a disulfide bond as indicated by the structure of IgG. Three different cDNAs (FSHα, Fc and FSHβ-Fc) were cloned into one plasmid with separate CMV promoters, generating the plasmid pCDNA4m-FSH-mono (Fig. 1A). The pCDNA4m-FSH-mono plasmids were transfected into CHO cells for KN015 expression and purification.

After purification, reducing and non-reducing SDS-PAGE (Fig. 1C) and SE-HPLC were used to determine purity and apparent molecular weight. With non-reducing SDS-PAGE, KN015 appeared as a single band of FSH-Fc/Fc heterodimers by Coomassie Brilliant Blue R250 staining (Fig. 1C, lane 1) with a molecular weight of about 82 KD. With reducing SDS-PAGE, KN015 appeared as three bands: FSHβ-Fc, FSHα and Fc (Fig. 1C, lane 3). The purity of KN015 used in our studies was ~98% as determined by SE-HPLC (data not shown).

Pharmacokinetics

To determine KN015 pharmacokinetics *in vivo*, 6-week-old female SD rats were each given a single subcutaneous injection of KN015 or Gonal-F at doses of 3.8 or 38 μg/kg body weight. Consistent with previous reports, the average elimination half-life ($t_{1/2}$) and peak serum concentration time (t_{max}) of Gonal-F were 7 and 5 h, respectively (Fig. 1D). The $t_{1/2}$ of KN015 in rats was around 84 h. The t_{max} was ~48 h after administration (Fig. 1D). This indicated that the Fc region had significantly prolonged the half-life of recombinant FSH.

Two Cynomolgus monkeys were administered KN015 with a single subcutaneous (sc.) injection of 3.8 or 38 μg/kg, and after injection, KN015 was slowly absorbed into the circulation with a mean t_{max} of 42 and 34 h, respectively (Fig. 1E). Moreover, the clearance of KN015 was dose dependent in Cynomolgus monkeys. The average $t_{1/2}$ of KN015 was about 215 h, which was 10-fold longer than that of Gonal-F in monkeys. There were proportional increases in C_{max} and drug exposures (AUClast) for the dose range studied. The absolute bioavailability of a single dose (38 μg/kg body weight) after sc. administration was about 100% (Fig. 1F).

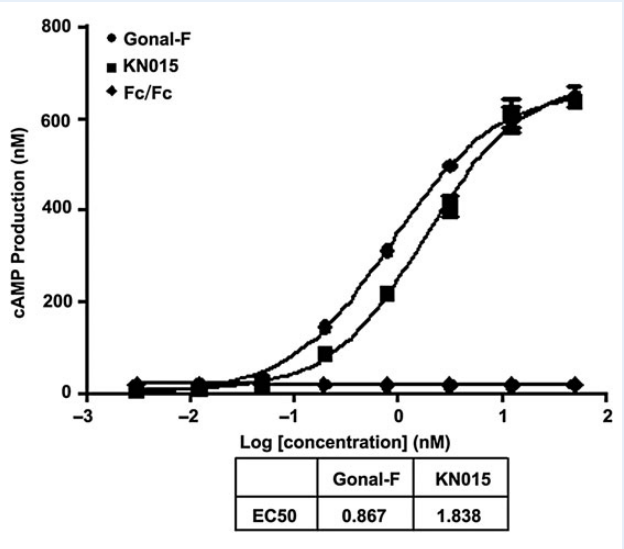
Overall, KN015 pharmacokinetics in both species was characterized by slow absorption and clearance after sc. administration. For both species, the half-life of KN015 after sc. administration was ~10- to 13-fold longer than that of Gonal-F (rhFSH).

cAMP induction *in vitro*

The FSH receptor (FSHR) is a G-protein-coupled receptor (GPCR). Upon its activation by FSH, FSHR induces cAMP synthesis that, in turn, triggers downstream signal transduction (Wayne *et al.*, 2007). In CHO cells stably bearing FSHR, the cAMP level in cell lysate dose dependently increased at 1 h after the addition of KN015 or Gonal-F, but not of Fc/Fc (Fig. 2). The EC50 value was 1.84 and 0.87 nM for KN015 and Gonal-F, respectively (Fig. 2). This showed the cAMP stimulation activity of KN015 was slightly lower (50%) than that of Gonal-F (Fig. 2), which indicated that KN015 was comparable with that of Elonva (Verbost *et al.*, 2011).

KN015 bioactivity for inducing oocyte maturation and cumulus cell-oocyte complex expansion *in vitro*

FSH or EGF-like factors can induce mouse oocyte GV breakdown (GVBD) and cumulus cell-oocyte complex (COC) expansion in culture (Eppig, 1979; Park *et al.*, 2004). To determine whether KN015 could stimulate COC expansion like native FSH, we performed COC *in vitro* expansion assays. Mouse COCs were isolated and cultured with vehicle, 1.25 pM Fc/Fc, KN015, or rhFSH, as well EGF as a positive control. Prior to culture, all COCs were intact and non-expanded. After culture for 16 h, all groups of treated COCs except the vehicle and Fc/Fc group, showed significant cumulus expansion when compared



	Gonal-F	KN015
EC50	0.867	1.838

Figure 2 cAMP production for Fc/Fc, KN015 and recombinant human FSH (Gonal-F) *in vitro*. Chinese hamster ovary (CHO) cells that expressed rat FSH receptors were incubated with serial dilutions of Fc/Fc, KN015 or Gonal F for 1 h at 37°C. Cells were lysed and cAMP levels were determined. Plotted points are means ± SD ($n = 6$).

with before culture (Fig. 3A). We then assessed mRNA expression levels of cumulus expansion-related genes that are known to be induced by FSH and EGF. As shown, the mRNA levels, encoding EGF-like factors, *Areg*, *Ereg* and *Btc* (intrafollicular paracrine factors that regulate ovulation) (Park *et al.*, 2004), were significantly up-regulated by KN015 and Gonal-F at comparable levels. Similarly, KN015 and Gonal-F were equally efficient for inducing the expression of genes (*Ptgs2, Has2, Ptx3* and *Tnfaip6*) directly required for COC matrix formation and expansion (Fig. 3B). Moreover, the GVBD rate of mouse oocytes enclosed in COC was counted after increasing concentrations of Fc/Fc, KN015 or Gonal-F treatment. We observed that the GVBD rate dose dependently increased in both KN015 and Gonal-F groups, but not in Fc/Fc-treated group. Furthermore, on a molar basis, the GVBD stimulation activity of KN015 is slightly lower (about 50%) than that of Gonal-F (Fig. 3C), consistent with the half of cAMP induction activity of KN015 relative to Gonal-F in Fig. 2.

KN015 bioactivity *in vivo*

In 2005, Low *et al.* have reported two long-life FSH-Fc fusion variants by oral or pulmonary delivery: single-chain FSH-Fc and heterodimer FSH-Fc, and the latter had higher bioactivity. In our study, we compared the *in vivo* bioactivity of KN015 and heterodimer FSH-Fc by a modified Steelman–Pohley assay (Steelman and Pohley, 1953). Heterodimer FSH-Fc had little bioactivity by subcutaneous injection of 21-day-old female SD rats (Fig. 4A), although it maybe had bioactivity by oral or pulmonary delivery. However, KN015 remarkably stimulated ovary weight gain (Fig. 4A and B).

The Steelman–Pohley assay (Steelman and Pohley, 1953) was based on the fact that FSH induces ovarian GC growth and triggers follicle development. We used a modified Steelman–Pohley assay (Steelman and Pohley, 1953), in which immature SD-rats received the same concentrations of KN015 or Gonal-F (0.19, 0.375, 0.75, 1.5, 3, 6 or 12 pmol/injection; $n = 8$/group), but with different administration frequencies. Only a single dose of KN015 was given, after which rats were sacrificed 84 h later. In contrast, seven injections of Gonal-F were given over 3 days followed by sacrifice at 12 h after the last injection.

Ovary weight gains and increased serum estradiol (E2) levels were observed in both groups of rats (Fig. 4B and C). These effects were dose dependent, although more pronounced changes were observed with KN015. Ovary weight reached a maximum of 166 mg after one injection of 1.5 pmol KN015, which was 1.8-fold higher than that after seven injections of 1.5 pmol Gonal-F (Fig. 4B). Estradiol (E2) levels reached 2460 pM after one injection of 3 pmol KN015, which was more than 4-fold higher than that with Gonal-F at the same concentration (Fig. 4C). HE staining of ovarian sections also showed significant dose-dependent follicle growth after Gonal-F or KN015 treatment (Fig. 4D). The greater potency of KN015 *in vivo* was presumably owing to its longer half-life.

Ovulation induction assays were performed using 26–28-day-old rats to compare the effects of KN015 and Gonal-F for supporting normal follicle growth. First, we tested for optimal timing for KN015 administration. hCG was injected at 72, 84 or 96 h after FSH treatment. This showed that maximal numbers of oocytes were ovulated when rats were injected with hCG at 84 h post-FSH treatment (Table II). Then, we treated rats with different doses of KN015 or Gonal-F for 84 h, followed by hCG injections (20 IU/rat for all FSH-treated groups). Rats received the same amount of KN015 or Gonal-F per injection (1, 2, 3, 6 or 12 pmol /injection; $n = 8$/group), but only a single injection of KN015 was given whereas four injections of Gonal-F were used over 3 days. Dose-dependent increases in the number of ovulated oocytes were observed in both KN015 and Gonal-F group, but the increases in KN015 group were more prominent and occurred at a much lower dose (Fig. 4E). The detailed data of the ovulation induction assay are summarized in Table III. Furthermore, images of COCs ovulated into the ampulla of the oviduct are shown in Fig. 4F. At a dose of 6 pmol per injection, few oocytes were observed in the oviducts of rats that received vehicle injections (Control), or a single injection of 6 pmol Gonal-F (Gonal-F × 1). In contrast, many normal COCs were observed in oviducts of rats that received multiple injections of 2.4 pmol GonalF (Gonal-F × n), or a single injection of 2.4 pmol KN015 (KN015 × 1). The oocyte quality was characterized by morphology (fragmentation or dark cytoplasm; Table III). In brief, KN015 showed a better ovulatory potency than Gonal-F (Fig. 4D and E) and the resulted oocytes have a comparable quality with those obtained from Gonal-F-treated rats (Fig. 4G and Table III).

KN015 induced FSH target genes expression in granulosa cells

FSH induces GC proliferation and activates the PKA and PI3K pathway, which is followed by the expression of multiple target genes, including *Fshr*, *Cyp19a1* and *Lhcgr* (Fan *et al.*, 2010). *Fshr* encodes for FSH receptors and its expression is induced by FSH for receptor expression on GC membranes in a positive feedback loop (Tano *et al.*, 1997). *Cyp19a1* encodes for aromatase, a key enzyme that converts androgen to estrogen. *Lhcgr* encodes for LH/hCG receptors and its expression is induced by FSH for these receptors expression on GC membranes in pre-ovulatory follicles. We assessed the effects of KN015 and Gonal-F

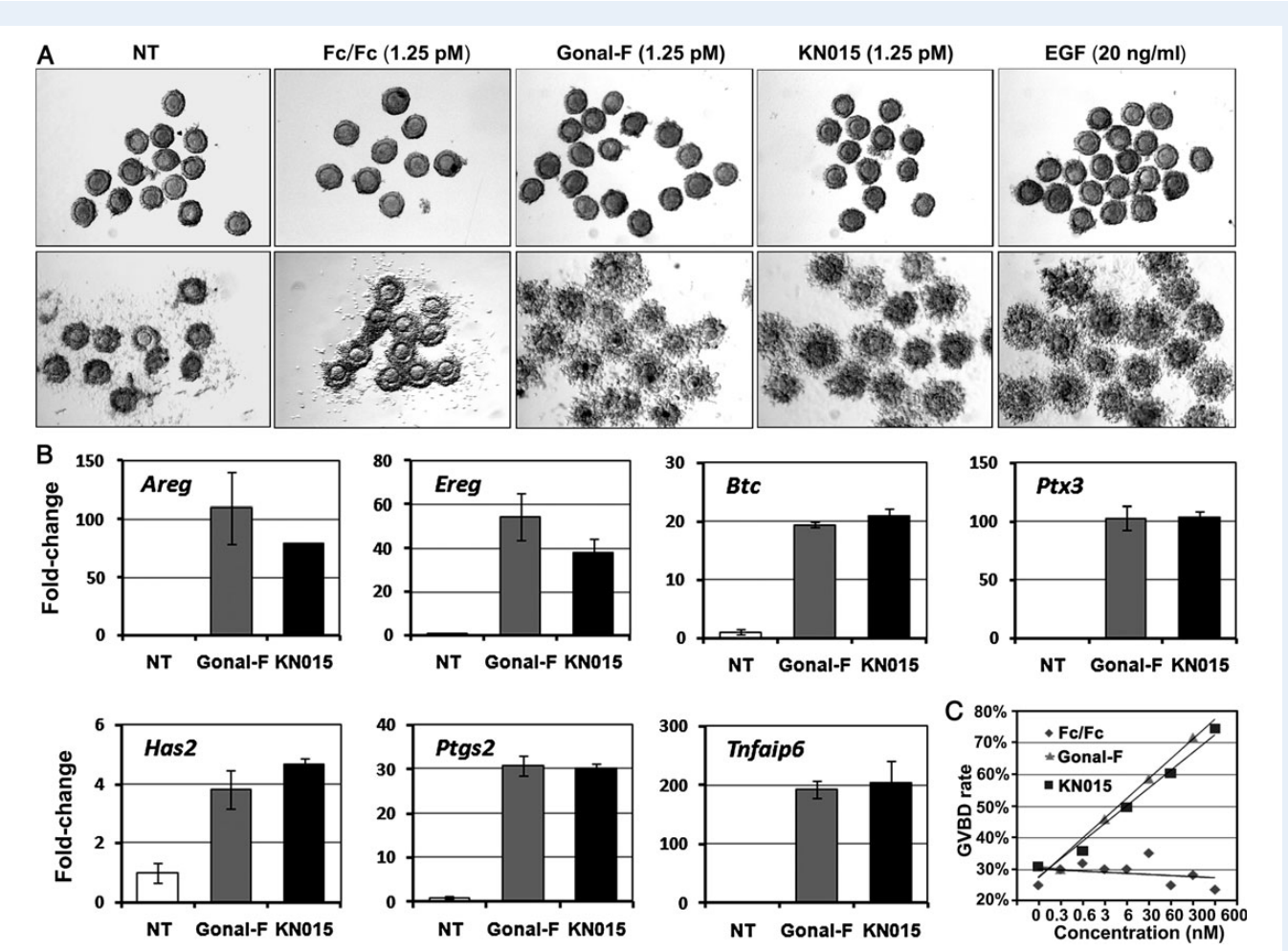


Figure 3 Germinal vesicle breakdown (GVBD) and cumulus oocyte complex (COC) expansion in rats induced by KN015. (**A**) *In vitro* COC expansion assay. Fully grown COCs were isolated from pregnant mare serum gonadotrophin-primed 21-day-old immature ICR mice and cultured in COC medium (20 COCs/50 μl medium). COC expansion was induced by treatment with 1.25 nM Fc/Fc, 1.25 nM Gonal-F, 1.25 nM KN015 or 20 ng EGF/ml overnight. NT: no treatment. Top row, 0 h; bottom row, after overnight incubation. (**B**) Expression of genes involved in COC expansion determined by real-time RT–PCR. Each gene was normalized to endogenous β-actin mRNA levels and the fold change was determined by setting the relative transcript level of the control sample (left-hand bar in each graph) to 1. A total of 50 fully grown COCs were used for real-time RT–PCR after culture with Gonal-F or KN015 (1.25 nM) for 4 h. (**C**) The GVBD rate of oocytes enclosed in COCs. Fully grown COCs collected from PMSG-injected day-21 mice were cultured in COC medium with Fc/Fc (0.3, 0.6, 3, 6, 30, 60, 300 or 600 nM), Gonal-F (0.3, 3, 30, 300 nM) or KN015 (0.6, 6, 60, 600 nM). GVBD rates were counted after 4 h in culture.

for inducing *Fshr*, *Cyp19a1* and *Lhcgr* mRNA expressions in rat GCs that were isolated from *in vivo*. GC *Fshr* mRNA levels were moderately increased after a single KN015 injection or multiple Gonal-F injections for 72 h (Fig. 5A). By comparison, a single Gonal-F injection did not induce *Fshr* expression, presumably due to its short half-life (Fig. 5A).

A single KN015 injection or multiple Gonal-F injections also increased *Cyp19a1* mRNA expression in GCs. Maximal *Cyp19a1* mRNA level was found at 72 h after using 12 pmol KN015 or Gonal-F. In addition, KN015 was more potent than Gonal-F for inducing *Cyp19a1* expression (Fig. 5B). Furthermore, both Gonal-F and KN015 induced significant *Cyp19a1* expression in cultured primary granulosa cells, in a dose-dependent manner (Fig. 5D). However, the increases in *Lhcgr* mRNA levels were not remarkable after either KN015 or Gonal-F treatment (Fig. 5C). It was possible that GCs had rapidly luteinized after administering a high dose of KN015 or Gonal-F.

FSH recruits a group of follicles to develop, which then produce estrogen. 3β-hydroxysteroid dehydrogenase (3β-HSD) is an enzyme that is required for androgen biosynthesis and is localized in theca cells of developing follicles and GCs of late-stage or pre-ovulatory follicles (Galas *et al.*, 2012). As shown by immunohistochemical staining (Fig. 5E), 3β-HSD expression was restricted to theca cells. Gonal-F and KN015 induced significant 3β-HSD expression in the GCs of antral follicles. Taken together, these results suggested that KN015 had bioactivities similar to that of Gonal-F for triggering pre-ovulatory follicle development.

KN015 mimics FSH by activating FSH downstream signal transduction pathways in GCs

FSH induces GC proliferation and estrogen synthesis by activating several signaling pathways, including PI3K and ERK1/2 pathways. To test

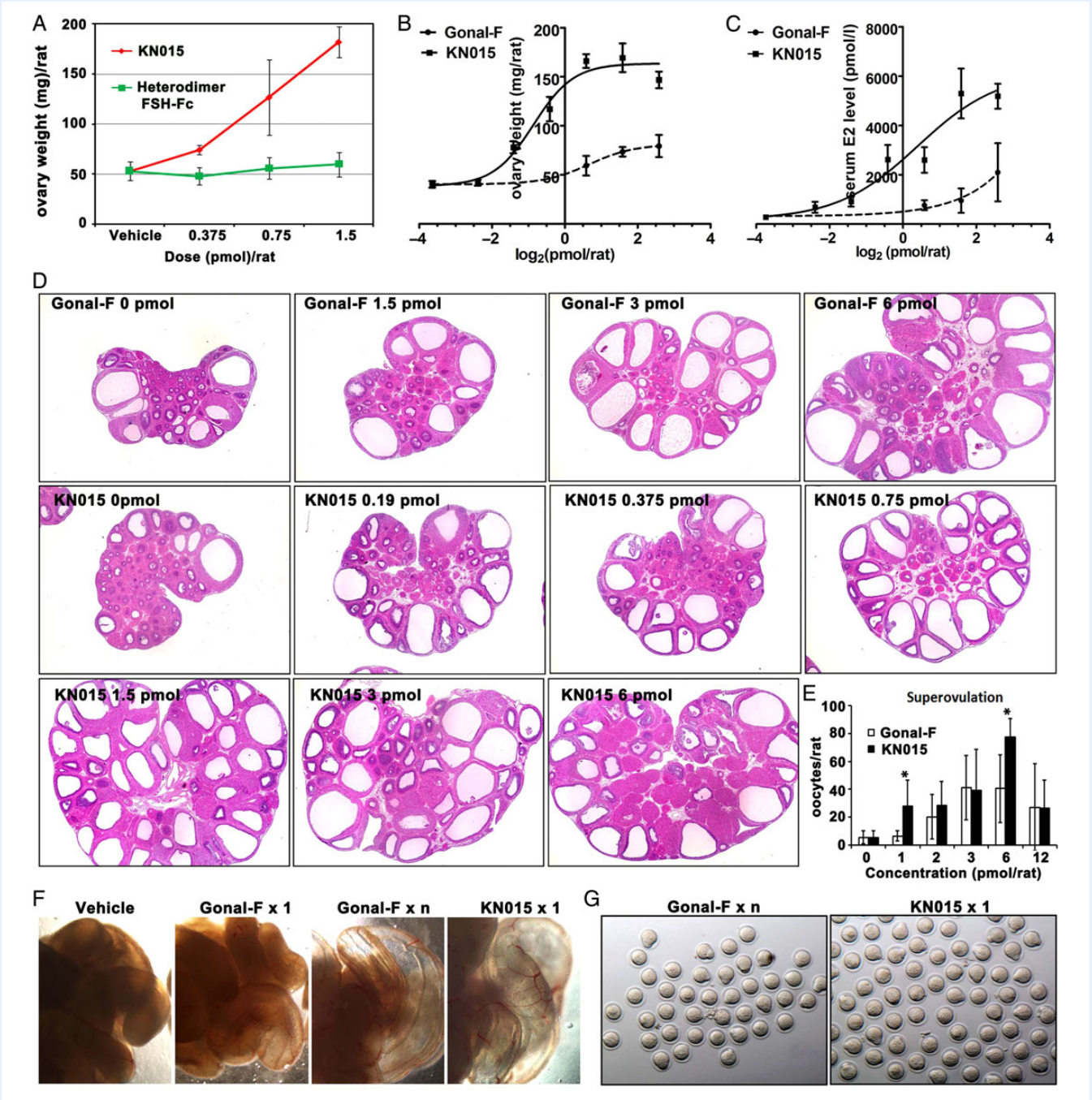


Figure 4 Pharmacodynamics of ovarian weight and serum estradiol (E2) levels in rats after a single injection of KN015 or several injections of Gonal-F. (**A**) Mean ovary weight comparison between KN015 and heterodimer FSH-Fc at 84 h both after a single injection in immature female SD rats. (**B** and **C**) Mean ovary weights (B) and serum estradiol (E2) levels (C) at 84 h after a single injection of KN015 or seven injections of Gonal-F administered at different doses. Seventy-five IU hCG/kg body weight was given daily on days 1, 2, 3 and 4. (**D**) HE staining of immature rats ovaries in an ovary weight gain assay. Immature rats (21-day-old) were treated with a single injection of KN015 or several injections of Gonal-F administered at different doses combined with hCG. (**E**) Numbers of oocytes induced by 20 IU of hCG at 84 h after a single injection of KN015 or several injections of Gonal-F (means ± SEM's; $n = 8$). Asterisks indicate significant differences between the respective KN015 and Gonal-F dose-matched groups ($P < 0.05$). (**F**) Images showing COCs in the ampulla of an oviduct in a representative animal from each treatment. (**G**) Images of oocytes ovulated by rats treated with Gonal-F or KN015.

whether these pathways were activated by KN015, GCs were cultured with Gonal-F or KN015 and collected at different time points. The phosphorylation levels of key components in the PI3K pathway, including AKT, FOXO1 and RPS6K, were significantly increased as early as 5 min after either KN015 or Gonal-F treatment (Fig. 5F). ERK1/2 phosphorylation was also up-regulated within 30 min of KN015 or Gonal-F

Table II Optimal timing of hCG application in super-ovulation assay.

Group	Time point of hCG injection	Dose (pmol)/rat (times)	Number of ova.
Control	72 h	0	3.3 ± 2.49
	84 h	0	4.3 ± 2.71
Gonal-F	72 h	6 (×1)	4.0 ± 3.61
	72 h	2.4 (×3)	26.6 ± 14.03
Gonal-F	84 h	2.4 (×4)	22.9 ± 11.66
	96 h	2.4 (×4)	7.5 ± 8.42
	72 h	2.4 (×1)	28.1 ± 14.09
KN015	84 h	2.4 (×1)	36.3 ± 24.13
	96 h	2.4 (×1)	10.6 ± 13.18

Numbers of ova/rat (means ± SEM's; $n = 8$) induced by hCG (20 IU) administered at different times (72, 84 or 96 h) after a single injection of KN015 or Gonal F or the first of several injections of Gonal-F given at 24 h intervals.

Table III The summary of ovulation induction assay after treatment with KN015 or Gonal-F.

Group	Dose (pmol)/rat (times)	Number of ova.	Number of abnormal (dark or degenerated) ova
Control	0	5.6 ± 4.81	0
	1 (×4)	6.6 ± 3.78	0.5 ± 1.07
	2 (×4)	20.1 ± 15.82	0
Gonal-F	3 (×4)	41.1 ± 23.10	0
	6 (×4)	40.5 ± 24.33	6.3 ± 16.50
	12 (×4)	27.3 ± 31.21	4.3 ± 6.11
	1 (×1)	28.1 ± 18.22	0
	2 (×1)	28.3 ± 17.36	0
KN015	3 (×1)	39.4 ± 29.15	0.4 ± 1.06
	6 (×1)	77.6 ± 12.73	1.5 ± 1.5
	12 (×1)	26.8 ± 19.64	3.3 ± 3.49

Numbers of ova (means ± SEM's; $n = 8$) induced by an injection at 84 h of 20 IU hCG in rats given different doses (1, 2, 3, 6 or 12 pmol/rat) of KN015 or Gonal-F. Rats were given a single dose of KN015 at 0 h but Gonal F was administered four times at 24 h intervals starting at 0 h.

treatment. Total ERK1/2 levels remained stable and were used as a loading control. These results suggested that KN015 could mimic FSH and activate FSH downstream signal transduction pathways in GCs.

KN015 induces GC proliferation and inhibits follicle atresia

Physiologically, FSH induces GC proliferation, follicle development and dominant follicle selection. FSH also inhibits antral follicle atresia as a major *in vivo* follicle survival factor (Shen *et al*, 2014). To determine whether KN015 had bioactivity for inducing GC proliferation and inhibiting apoptosis, we used immunohistochemical staining for phosphorylated histone H3 at serine-9 (pHH3) and a TUNEL assay. As shown in Fig. 6A, the numbers of pHH3-positive GCs were significantly increased after KN015 or Gonal-F treatment. Also, *in vitro* MTT assays of cultured granulosa cells showed KN015 stimulates granulosa cell proliferation better than Gonal-F as culture time prolonged to 48 h (Fig. 6B). This indicated that KN015 could induce GC proliferation as could Gonal-F. TUNEL assay results showed that the numbers of atretic follicles were reduced with increasing doses of KN015 or Gonal-F (Fig. 6C). With increasing doses, the mean number of atresia follicles per section was reduced from 24 to 12 with Gonal-F and from 20.7 to 8.7 for KN015 (Fig. 6D).

Discussion

Recombinant hFSH has been used for years for infertility treatments (e.g. IVF). However, due to its short half-life, daily injections are necessary to maintain FSH levels above the threshold required to support multiple follicle growth. This short half-life is due to the inherent properties of FSH and primarily filtration is through the kidneys due to its relatively small molecular size and endocytosis (Tang *et al.*, 2004). During recent decades, significant efforts have been made to improve the half-life of FSH. It was hypothesized that one way of extending the $t_{1/2}$ of FSH would be to reduce glomerular filtration by increasing the molecular weight and charge of the molecule via the introduction of additional glycosylation (Ben-Menahem and Boime, 1996). As examples, introducing additional glycosylation through either CTP fusion at a subunit at the C-terminus of FSH-β or inserting additional glycosylation at the N-termini of both subunits (Sugahara *et al.*, 1996; Perlman *et al.*, 2003), C-terminal Fc fusion was also attempted (Low *et al.*, 2005) with FSH activity significantly reduced. Additional glycosylation could only marginally increase the half-life of FSH, which leads to the generation of Elonva, the only commercially available long-acting FSH. One injection of Elonva used at dose of >10-fold higher than that for WT FSH can cover the first 7 days of treatment followed by a supplemental injection of WT FSH. However, this initial high dose can cause some unexpected side effects, for example the incidence of ovarian hyper-stimulation Syndrome (OHSS) in clinical phase III was 1–2% higher than that of rhFSH (Devroey *et al.*, 2009; Group TCAES, 2010). Thus, developing a better long-acting FSH is warranted.

The Fc fragment of an IgG antibody is the primary reason for its considerably long half-life of several weeks in the body. Fc fusion with an active peptide or proteins is a common means to improve the half-lives of biotherapeutics. An Fc fragment can exert its beneficial effects in two ways: by increasing the size of a fused protein by 55 KD and by rescuing the fused partner from lysosome digestion via binding to FcRn in a pH-dependent manner (Tabrizi *et al.*, 2006). Low *et al.* (Low *et al.*, 2005) developed two long-life FSH-Fc fusion variants by oral or pulmonary delivery: single chain FSH-Fc and heterodimer FSH-Fc. The single chain FSH-Fc is a homodimer of FSH-α-β-Fc (FSHα, FSHβ and Fc ligated to form a contiguous fusion) whereas the heterodimer FSH-Fc is composed of FSH-α-Fc and FSH-β-Fc (the α and β subunit linked to separate arm of the Fc fragment), the latter of which has higher bioactivity. In our study, we found that heterodimer FSH-Fc had little bioactivity in stimulating ovary weight augment (Fig. 4A), although it has been reported to have bioactivity by oral or pulmonary delivery. Although other efforts have failed, we still feel it should be possible to extend the half-life of FSH with good activity by carefully designing the fusion product.

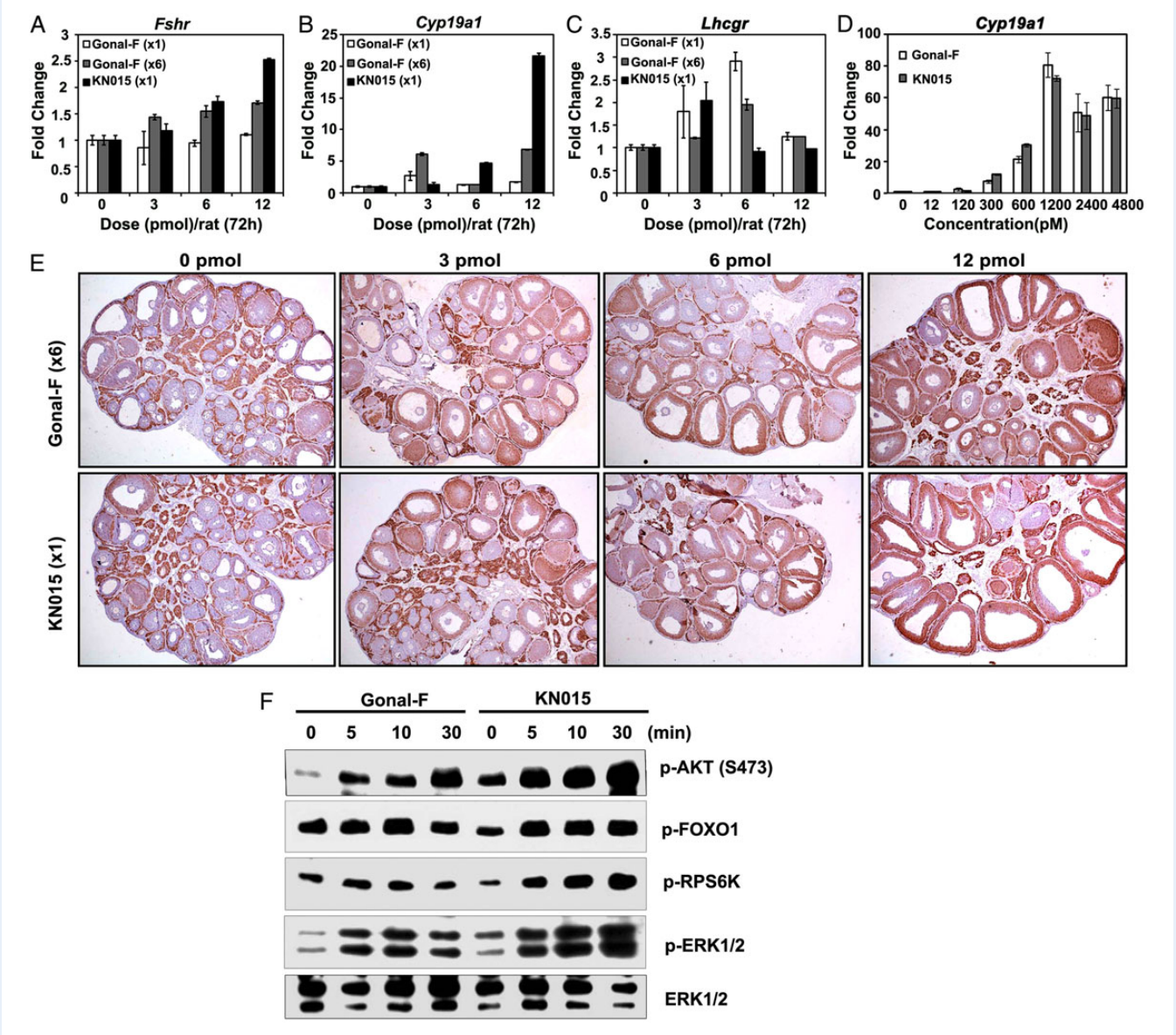


Figure 5 FSH target gene expression and downstream signaling pathway induced by KN015 *in vivo*. (**A**–**C**) Real-time RT–PCR results for *Fshr* (A), *Cyp19a1* (B) and *Lhcgr* (C) mRNA expression in ovaries of 21-day-old rats at 72 h after treatment with KN015 or Gonal-F. $n = 6$ for each treatment. (**D**) Real-time RT–PCR result for *Cyp19a1* mRNA expression in primary granulosa cells cultured for 24 h after treatment with different doses of KN015 or Gonal-F. The indicated genes in D and E were normalized to endogenous β-actin mRNA levels and the fold change was determined by setting the relative transcript level of the control sample (left-hand bar in each graph) to 1. (**E**) Immunohistochemical staining for 3β-HSD (brown) in ovaries of 21-day-old rats at 72 h after treatment with a single injection of KN015 or six injections of Gonal-F. $n = 6$ for each treatment. (**F**) Western blot results showing that phosphatidylinositide 3-kinase (PI3K) and extracellular receptor kinase (ERK) pathways were activated. The key components of PI3K (AKT, FOXO1 and RS6K) and ERK1/2 were phosphorylated by 30 min when GCs were treated with Gonal-F or KN015 (1.2 nM).

Biochemical and mutagenesis data have indicated that the C-terminus of the FSH-α subunit is critical for binding to FSH receptors, as demonstrated by the crystal structure of an FSH-FSHR complex (Fan and Hendrickson, 2005; Jiang *et al.*, 2012). We speculated that either the single chain FSH-Fc or the heterodimer FSH-Fcin Low's paper (Low *et al.*, 2005) has low bioactivity because the FSHα was fused with Fc or FSHβ, resulting in steric hindrance when binding FSHR. On the basis of these considerations, KN015 was designed as a heterodimeric FSH-Fc/Fc fusion protein by fusing only the FSH-β subunit to an IgG1 Fc fragment while leaving the α subunit alone. A 15-amino acid linker peptide was inserted between FSH-β and Fc to ensure flexibility and minimize the impact on subunit assembly and FSH function.

Our results clearly indicated that the receptor binding and stimulation activity of KN015 were well preserved. Generally, an ovary weight augmentation assay is conducted with dosing for 72 h using the Steelman–Pohley assay. Because the half-life of KN015 was much longer than that of Gonal-F, the effective timing for the ovary weight gain assay and ovulation induction assay was investigated further. We determined that both

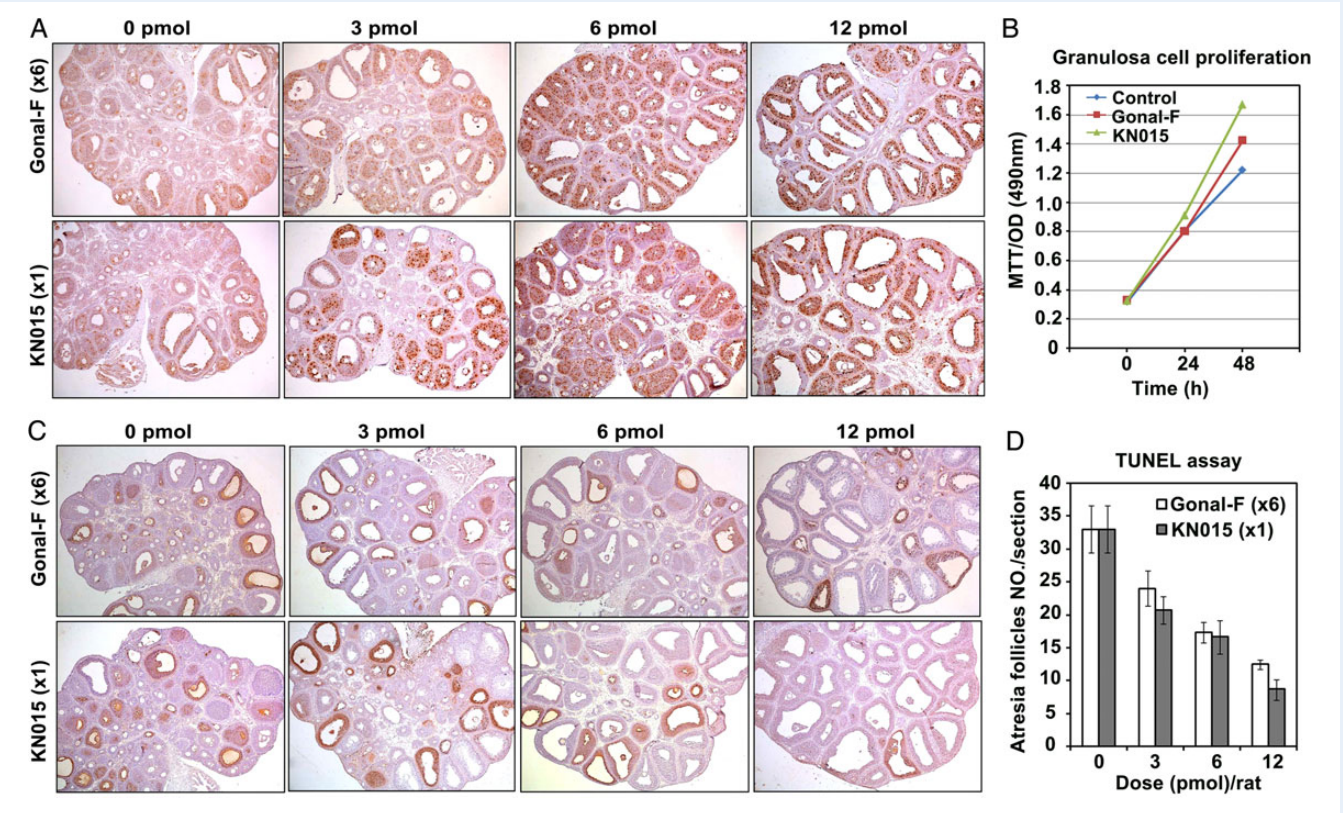


Figure 6 Inducement of granulosa cell proliferation and inhibition of follicle atresia by KN015. (**A**) Immunohistochemical staining for phospho-histone H3 (pHH3) showing granulosa cell proliferation in ovaries after a single injection of KN015 or six injections of Gonal-F. $n = 6$ for each treatment. (**B**) The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay showing the *in vitro* granulosa cell proliferation induced by (1200 pM) KN015 or Gonal-F. This experiment was done in triplicate. (**C**) TUNEL assay showing granulosa cell apoptosis and follicle atresia in ovaries at 72 h after a single injection of KN015 or six injections of Gonal-F. $n = 6$ for each treatment. (**D**) Numbers of atretic follicles were counted per section (means $\pm$ SEM's; $n = 4$).

Gonal-F and KN015 treatment achieved maximum ovary weights at 84 h after sc. administration (data not shown). Similarly, KN015 induced maximum ovulation induction by 84 h whereas Gonal-F achieved this at 72 h (Table II). This difference was most likely due to the slower absorption of KN015 as indicated by pharmacokinetic analysis. KN015 had a much longer half-life, about 10-fold longer than FSH of ~84 h in rats and ~200 h in Cynomolgus monkeys. To exclude the possibility that our Fc fusion might alter the physiological function of FSH, we conducted a series of experiments to compare WT FSH and KN015 in detail.

FSH induced GC proliferation and pre-antral follicle development into antral follicles, which resulted in ovary weight gains. One dose of KN015 was significantly more effective than several injections of rhFSH with regard to ovary weight gain as KN015 maintained a better effective concentration. A similar trend was found with a rat ovulation induction assay. To better understand the mechanism of KN015 at the cellular and molecular levels, additional *in vitro* experiments were performed.

It has been reported that FSH could induce COC expansion and GC proliferation and prevent follicle atresia. As expected, KN015 had comparable activity in these respects when compared with Gonal-F. Relevant gene expression levels were also determined after KN015 or Gonal-F treatment. There were no obvious differences with regard to the genes that were regulated and their expression levels. The canonical signaling pathway for FSH is through FSHR, which is a GPCR that induces cAMP production. We found that KN015 could induce cAMP synthesis in FSHR bearing CHO cells, albeit at a slightly lower potency. To better understand KN015 signaling activity, a non-canonical pathway that involved PI3K and ERK1/2 was also investigated. From these results, it was clear that KN015 could induce the phosphorylation of key components of the PI3K pathway and ERK1/2 in a similar manner as Gonal-F in GCs.

In summary, the presence of an Fc fragment fused to FSH did not significantly interfere with its secretion, assembly, FSHR binding signal transduction and bioactivity *in vitro*. Moreover, KN015 had a longer half-life and higher activity *in vivo*. Its half-life of 200 h in monkeys provides significant advantages relative to rhFSH in terms of tolerance and convenience for infertility therapy. Collectively, our results suggest that KN015 may have great potential for therapeutic applications.

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Authors' roles

T.X., and H.-Y.F., K.-P.G. and Y.-L.Z. are involved the conceptualization of study design. K.-P.G. constructed the plasmid, purified the KN015 and

determined its molecular weight. Y.-L.Z. performed ovary weight gain assay, ovulation induction assay, oocyte maturation assay, RT–PCR, staining and wrote the manuscript. S.-Y.J. and X.-M.L. performed the ovary weight gain assay, COC expansion assay and western blot. P.W. and J.W. performed the cAMP assay. L.G. and T.-Q.J. conducted the pharmacokinetics in SD rats and monkeys, respectively. H.-Y.F. made the figures and revised the manuscript. T.X. revised the manuscript.

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Conflict of interest

None declared.

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